(ii) inserting a differentiated mammalian donor cell, or the nucleus of said cell, into the oocyte under conditions suitable for the formation of a nuclear transfer unit so that a nuclear transfer unit is formed, wherein said oocyte and said differentiated cell are from different mammalian species;

(iii) activating the resultant nuclear transfer unit; and

(iv) culturing the activated nuclear transfer unit to produce a multicellular structure.

REMARKS

This amendment is responsive to the Final Office Action dated September 10, 2002. The claims are rejected under 35 U.S.C. §103(a) as being obvious in view of Wolfe *et al.*(1990), taken with Collas et al. (1994). To expedite prosecution, independent claim 36 is amended to specify that the nuclear transfer unit having genomic DNA of one mammalian species and mitochondria of a different mammalian species that is produced by the claimed method is capable of giving rise to a multicellular structure of at least about 50 cells when cultured under appropriate conditions. Support for the amendment is found in the specification, for example, at page 11, lines 14-15 ("to a size of at least about 50 cells"), and in Example 1, page 30, lines 19-22 ("NT units reaching the desired cell no., i.e., about 50 cell number, were mechanically removed from the zona and used to produce embryonic cell lines").

The Applicants submit that the amendment does not raise new issues for examination, in view of examined claim 57 which recites producing a multicellular nuclear transfer unit of "about 2 to 400 cells," and respectfully request entry of the amendment.

Regarding the rejection of claims 36-87 under 35 U.S.C. §103(a) as being unpatentable over Wolfe *et al.*, taken with Collas et al.:

Claims 36-87 were rejected under 35 U.S.C. 103(a) as being unpatentable over Wolfe *et al.* (1990) in view of Collas et al. (1994).

The Applicants submit that the disclosure of Wolfe *et al.* (1990), in combination with that of Collas et al. (1994), would not have suggested to a person of ordinary skill in the art that transfer of the nucleus of a differentiated cell into an oocyte of a different species would have reasonably beeen expected to result in production of a nuclear transfer unit that is capable of giving rise to a multicellular structure of at least about 50 cells. In support of this position, the Applicants respectfully submit the declaration of Robert P. Lanza, M.D., pursuant to 37 C.F.R. § 132.

As set forth in the attached Curriculum Vitae, Dr. Lanza is Vice-President of Medical & Scientific Development at Advanced Cell Technology, the assignee of the present invention; he has conducted and directed research in the areas of tissue engineering and cell and tissue transplantation, including xenogeneic transplantation; he has published numerous articles relating to cloning, including interspecies nuclear transfer; and he is a co-inventor of pending patent applications relating to intra- and inter-specific cloning.

As stated in Dr. Lanza's declaration, in August 1996, at the time of filing of U.S. Patent Application No. 08/699,040, the parent of the present application, the discovery of the present application that the nucleus of a differentiated human cell could be transferred into an enucleated bovine oocyte to produce a nuclear transfer embryo capable of undergoing cleavage and developing into a multicellular structure was an unexpected and surprising result. At that time, no other research group had disclosed or suggested that a multicellular

embryo could be successfully produced by inter-specific nuclear transfer using a differentiated nuclear donor cell.

In the late 1980s and early 1990s, methods were developed for cloning by nuclear transfer that used undifferentiated embryonic cells, i.e., blastomeres, as nuclear donor cells. At that time, it was recognized that the embryonic development involves many complex interactions between the nuclear chromatin and factors in the recipient oocyte that were necessary for regulated activation of transcription of genes of the embryonic genome (Nothias et al., 1995, J. Biol. Chem., 270:22077-80), and persons of skill in ther art generally recognized that as donor embryonic cells differentiated, they lost their capacity to direct embryonic development and blastocyst formation. As discussed in the Collas et al. reference, the restricted developmental potential of nuclei from differentiated cells was thought to be due, at least in part, to changes related to DNA replication and gene expression, and failure to reactivate genes that became transcriptionally inactive during differentiation. It was proposed that incomplete DNA replication could result from asynchrony in the cell cycle of the donor nucleus and recipient oocyte, or from changes in the pattern of transcriptionally active and inactive chromatin and their timing of replication. (see Collas et al., p. 266).

The "remodeling" or "reprogramming" of a nucleus of a differentiated cell so that it is capable of successfully directing embryogenesis following transfer into an oocyte was thought to require similar reactions and protein exchanges between the recipient ooplasm and the donor chromatin (for example, see Prather et al., J. Reprod. Fertil. Suppl., 1990, 41:125-34). However, it was recognized that reprogramming following inter-specific nuclear transfer was even more complex and unpredictable than reprogramming associated with intra-specific nuclear transfer, because the complex reactions and interactions between the nuclear chromatin and oocyte components that are required for embryonic development

would have to occur across the evolutionary divide between the two species. It was recognized that evolutionary divergence of the structures of the nuclear chromatin relative to the corresponding oocyte components could result in structural incompatibilities that could inhibit or alter reactions required for successful embryogenesis. Species-specific variations in the timing of synthesis or degradation and/or in the concentrations of chromatin binding sites, oocyte components, other structural proteins, and metabolic enzymes, were seen as providing an additional layer of complexity to embryonic development following inter-specific nuclear transfer. At the time the parent application was filed, it was known that the complement of proteins that is initially produced by an embryonic genome varies from species to species (Crosby et al., J. Reprod. Fertility, 1988, 82:769-75; Barnes et al., Molecular Reproduction and Development, 1991, 29:117-9; and Connover et al., Dev. Biology, 1991, 147:403-14). In addition, it was known that the stage of embryonic development at which transcription of embryonic genes begins, referred to as the "maternal to embryonic transition" (MET), also varies from species to species; e.g., transcription of genomic DNA begins in in murine and rat embryos at the 2-cell stage, in bovine embryos at the late 4- to early 8-cell stage, in human embryos at the 4- to 8-cell stage, and in sheep embryos at the 8- to 16-cell stage (see Telford et al., Molecular Reproduction and Development, 1990, 26:90-100). Species-specific incompatibilities between the oocyte-derived mitochondria and proteins expressed by nuclear genes were regarded as another source of species-specific discordance that could interfere with the successful development of embryos produced by cross-species nuclear transfer. Mammalian mitochondrial DNA codes for 13 enzymes that mediate oxidative phosphorylation, 22 tRNAs, and two rRNAs (Smith et al., J. Reprod. Fertil. Suppl. 48:31-43, 1993). Kenyon et al. showed that oxidative phosphorylation is impaired in cells with human genomic DNA and mitochondria of

orangutan, New World monkeys, or lemurs, but not in cells with human genomic DNA and mitochondria of gorillas or chimpanzees (Proc. Nat. Acad. Sci. U.S.A., 94:9131-9135, 1997).

In view of the above-discussed reasons why the success of inter-specific nuclear transfer was uncertain, the report by Wolfe et al. that blastocysts could be generated following inter-specific transfer of blastomere nuclei into recipient oocytes was initially regarded as significant, because it suggested that evolutionary divergence does not prevent interactions between an undifferentiated nucleus of one species and the ooplasm of a different species that mediate successful development of a nuclear transfer embryo. However, doubts as to whether the 1990 reference actually demonstrated production of a blastocyst directed by a nucleus of one species after transfer into an oocyte of a different species were raised by Wolfe et al. (Biol. of Reprod., 1994, 50 (Suppl. 1) p. 72, copy attached), a scientific article that was written by three of the same authors as the Wolfe et al. (1990) reference that was cited as prior art. The 1994 Wolfe et al. reference stated that the blastocysts reported in the 1990 reference as having been obtained by interspecific nuclear transfer may have actually been the result of parthenogenetic development of nucleated demi-oocytes, because the nuclear transfer methods described in the 1990 reference did not include checking to verify that the recipient oocytes were successfully enucleated. The 1994 Wolfe et al. reference also stated that attempts to reproduce the production of blastocysts by interspecific nuclear transfer as described in the 1990 reference were unsuccessful, and concluded that the interspecies nuclear transfers were incapable of developing into blastocysts. In view of the 1994 Wolfe et al. reference, persons of ordinary skill in art would not have regarded the report of the 1990 Wolfe et al. reference that interspecific nuclear transfer leads to successful embryonic development as valid and convincing.

The 1990 Wolfe et al. reference described performing interspecific nuclear transfer using donor nuclei of undifferentiated blastomeres; therefore, even if the results reportd in the

1990 Wolfe et al. reference had been valid, the method used by Wolfe et al. was qualitatively different from the claimed invention, which performs inter-specific nuclear transfer using donor nuclei of differentiated cells. As discussed in Dr. Lanza's declaration, at the time the parent application was filed, nuclear transfer using undifferentiated donor nuclei was a known and routinely practiced cloning technique, and persons skilled in the art knew that the chromatin of embryonic donor cells is in an embryonic configuration that is capable of directing embryogenesis with relatively high efficiency. In contrast, successful cloning by nuclear transfer using donor nuclei of differentiated cells was at that time known to be a relatively new and much more problematic methodology. Persons skilled in the art knew that the chromosomes of a differentiated donor cell are in a non-embryonic state, and must undergo significant reprogramming in order for successful embryonic development to occur. The molecular basis for reprogramming a differentiated cell nucleus following nuclear transfer into a recipient oocyte was then and remains today poorly understood. As discussed above, successful reprogramming of a differentiated donor cell nucleus in an oocyte of a different species was seen as involving the complex reactions and interactions between the chromatin of the differentiated cell and oocyte components that are required for reprogramming a nulceus of like species, plus additional, species-specific and time-sensitive reactions and interactions between the donor chromatin and the components of the recipient oocyte that are unique to reprogramming a nucleus following inter-specific transfer. In view of the combined effects of structural incompatibilities between the proteins of the nuclear chromatin and oocyte components that could interfere with reprogramming, together with species-dependent asynchronies in the timing and pattern of embryonic gene expression, and discordance between genome- and oocyte-derived mitochondrial proteins, persons of ordinary skill in the art at the time the parent application was filed could not have predicted whether or not interspecies nuclear transfer using differentiated donor cell nuclei would lead

to successful embryonic development and production of blastocysts. Therefore, even if the 1990 Wolfe et al. reference had convincingly reported successful embryonic development following inter-specific nuclear transfer using donor nuclei of undifferentiated blastomeres, that result would not have suggested to a person of ordinary skill in the art that inter-specific nuclear transfer using donor nuclei of differentiated cells would reasonably be expected to result in embryonic development.

The deficiencies of the 1990 Wolfe et al. reference would not have been remedied by the Collas et al. reference. Collas et al. showed transfer of a nucleus of a differentiated somatic cell into an enucleated oocyte of the same species cold lead to production of a blastocyst; but the 1990 Wolfe et al. reference in combination with the Collas et al. reference would not have suggested to a person of ordinary skill in the art that inter-specific nuclear transfer using differentiated donor cell nuclei would lead to successful embryonic development. As discussed above, the 1994 Wolfe et al. reference stated that the methodology of the 1990 Wolfe et al. reference was flawed, and that attempts to reproduce the production of blastocysts by inter-specific nuclear transfer were unsuccessful, so that one of ordinary skill in the art would not have relied on the teachings of the 1990 Wolfe et al. reference.

Moreover, at the time the parent application was filed, the process of reprogramming a differentiated donor nucleus following inter-specific nuclear transfer so that embryogenesis can proceed was regarded as involving complex, unpredictable interactions and reactions between the donor nuclei and oocytes and properly regulated transcription of genes of the embryonic genome, as discussed above. The Collas et al. reference only describes the reprogramming of a cell nucleus in an oocyte of the same species. In view of the additional complexities and reasons for uncertainty believed to be associated with reprogramming inter-specific nuclear transfer, the results reported by Collas et al., alone or in combination with

Wolfe et al., would not have enabled a person of ordinary skill in the art to predict whether successful embryonic development would occur following transfer of the nucleus of a differentiated cell into an oocyte of a different species. Therefore, the successful operation of the claimed invention would not have been obvious to a person of ordinary skill in the art at the time the parent application was filed. In view of the foregoing, the Applicants submit that the invention could not have been prima facie obvious at the time the application was filed, and respectfully request that the rejection of claims under 35 U.S.C. §103(a) be withdrawn.

All issues raised by the Office Action dated September 10, 2002, have been addressed in this Reply. If the Examiner has any further questions or issues to raise regarding the subject application, it is respectfully requested that she contact the undersigned so that such issues may be addressed expeditiously.

Respectfully submitted,

Date: March 10, 2003

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APPENDIX

The changes in claim 36 made by the above amendment are shown below:

36. (Amended) A method for producing a nuclear transfer unit having genomic DNA of one mammalian species and mitochondria of a different mammalian species, and being capable of giving rise to a multicellular structure of at least about 50 cells when cultured under appropriate conditions, comprising:

(i) removing the genomic DNA from a mammalian oocyte;

(ii) inserting a differentiated mammalian donor cell, or the nucleus of said cell, into the oocyte under conditions suitable for the formation of a nuclear transfer unit so that a nuclear transfer unit is formed, wherein said oocyte and said differentiated cell are from different mammalian species;

(iii) activating the resultant nuclear transfer unit; and

(iv) culturing the activated nuclear transfer unit to produce a multicellular structure.

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EMBRYOS PRODUCED BY THE TRANSFER OF CAPRINE NUCLEI TO ENUCLEATED BOVINE OOCYTES ARE CAPABLE OF CLEAVAGE BUT DO NOT DEVELOP TO BLASTOCYSTS

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The objective of this study was to investigate the developmental capacity in an in vivo culture system of embryos produced by the transfer of nuclei from early caprine morulae to enucleated bovine oocytes. Previous studies (Theriogenology 33: 350, 1990; Proc. Symp. Cloning Mammals by Nuclear Transplantation, Ft. Collins, CO: 42-44, 1992) utilizing bisected bovine oocytes as nuclear recipients have indicated low frequency (1-2%) development to blastocysts of interspecies nuclear transfer embryos. This may have been due to parthenogenetic development of nucleated recipient demi-oocytes. In the present study, partial zona dissection, blastomere fusion and agar embedding were performed as previously described (Theriogenology 37: 5-15, 1992), except that enucleation was performed under fluorescence following a 30 min incubation in 5 µg/ml Hoechst 33342. Donor caprine and control bovine morulae were collected non-surgically on day 5.5 post coitus from superovulated/bred females. Following fusion and embedding, reconstituted embryos were transferred surgically to the ligated oviducts of progesterone-implanted Rambouillet ewes for 7 days. Developmental stage and nucleation were assessed following recovery. These results are shown below.

Embryo sp. / Oocyte sp.	# of Pairs	Fused (%T)	Recovered (%T)	Deg (%R)	1C (%R)	2-8C (%R)	9-16C (%R)	Mo (%R)	Bl (%R)
Caprine / Bovine	160	134 (83.8)	145 (90.6)	89 (61.4)	26 (17.9)	26 (17.9)	4 (2.8)	0	0 [a]
Bovine / Bovine	70	57 (81.4)	30 (42.9)	21 (70)	2 (6.6)	1 (3.3)	1 (3.3)	0	4 [b] (13.3)

%T = % transferred to recipient host
%R = % recovered from recipient host
a,b Different superscripts indicate significant difference by chi-square analysis ($p<0.05$)

Multicellular interspecies embryos recovered were observed under fluorescence to differentiate normal cleavage from fragmentation. Of thirty 2- to 16-celled embryos recovered from intermediate hosts, one embryo, which appeared to contain 12 cells, demonstrated 6 nuclei. Of the remaining multicellular embryos, 6 had two visible nuclei, 5 had a single nucleus, and 4 had no visible nuclei. The remaining 14 embryos were degenerating, and nucleation could not be determined. Therefore, most of the apparent development was due to fragmentation and not to nuclear cleavage. These results demonstrate that some interspecies nuclear transfer embryos are capable of one or more cleavage divisions, indicating some functional compatibility between the nuclear and cytoplasmic components of these species. However, these interspecies nuclear transfer embryos are not capable of normal development to blastocysts.

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Embryos produced by the transfer of caprine nuclei to enucleated bovine oocytes are capable of cleavage but do not develop to blastocysts.
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Minireview

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Regulation of Gene Expression at the Beginning of Mammalian Development*

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Life begins for most animals when sperm fertilizes an egg to form a zygote. What do we know about the mechanisms that activate zygotic gene expression in mammals and thereby turn on the developmental program? Historically, answers to this question have relied heavily on studies done with fertilized eggs from frogs and flies (1) and on studies of gene expression in animal viruses and differentiated cells. Even with the most convenient and well characterized mammalian developmental system, the mouse, the major impediment to studies on zygotes is their limited availability (~30/female) and small size (100–1000 times smaller than those from frogs or flies). One solution to this problem has been to inject unique DNA sequences in the form of plasmid DNA into the nuclei of oocytes and cleavage stage embryos. Replication and expression of genes encoded in extrachromosomal DNA respond to the same signals that regulate these functions in cellular DNA (2). They require specific cis-acting regulatory sequences and the trans-acting proteins that activate them and occur only when the host cell executes the same function with its own genome. These results, taken together with analyses of endogenous gene expression and results from nuclear transplantation studies, reveal several novel features of zygotic gene expression at the beginning of mammalian development (Fig. 1). These include the presence of a time-dependent mechanism for regulation of transcription and translation, activation of a chromatin-mediated repression of promoter activity, the developmental acquisition of enhancer-dependent and TATA-box-dependent transcription, and identification of transcription factors that are active at the onset of mammalian development.

Activation of Zygotic Gene Expression in Mice

A growing mouse oocyte, arrested at diplotene of its first meiotic prophase, transcribes and translates many of its own genes, thereby producing a store of proteins sufficient to support development to the 8-cell stage (3, 4) (Fig. 1). Transcription of injected genes at this stage requires specific promoter elements, such as binding sites for Sp1, E2F, and TBP[1] (5–7), or an oocyte-specific promoter such as ZP3 (8, 9). When an oocyte matures into an egg, it arrests in metaphase of its second meiotic division where transcription stops and translation of mRNA is reduced (10). Fertilization of the egg triggers completion of meiosis and formation of a 1-cell embryo containing a haploid paternal pronucleus derived from the sperm and a haploid maternal pronucleus derived from the oocyte. Each pronucleus then undergoes DNA replication before entering the first mitosis to produce a 2-cell embryo containing two diploid "zygotic" nuclei, each with a set of paternal and a set of maternal chromosomes.

Formation of a 2-cell mouse embryo marks the transition from maternal gene to zygotic gene dependence. Maternal mRNA degradation is triggered by meiotic maturation and ~90% completed in 2-cell embryos, although maternal protein synthesis continues into the 8-cell stage (11–13). Zygotic gene activation (ZGA) is recognized by the sensitivity of protein synthesis to α-amanitin, a specific inhibitor of RNA polymerases II and III. ZGA involves synthesis of about 40 proteins (14) and is not evident until 2–4 h after completion of the first mitosis, concurrent with S-phase in 2-cell embryos (13–18). Zygotic protein synthesis increases 8–10 h later during G_2-phase (15), suggesting that transcription of zygotic genes by RNA polymerase II occurs in two phases (Fig. 1), an early phase that is restricted to 2-cell embryos and a much stronger late phase that is required for further development (14, 16, 18).

Zygotic Clock

One of the most striking features of early ZGA is that its onset is delayed by a time-dependent mechanism referred to as the zygotic clock rather than by a particular cell cycle event. Early ZGA in the mouse occurs ~24 h after fertilization, regardless of whether or not the 1-cell embryo has completed S-phase and formed a 2-cell embryo (13, 16, 17). In contrast, late ZGA does not occur without formation of a 2-cell embryo (19). Thus, when 1-cell embryos that have not yet formed pronuclei are incubated in aphidicolin, a specific inhibitor of replicative DNA polymerases, they arrest development as they enter S-phase, but early ZGA still occurs at the time when they would have become 2-cell embryos (Fig. 1). Expression of plasmid-encoded genes injected into these arrested 1-cell embryos also is delayed until ZGA (17, 20).[2]

Although the bulk of both transcription and translation of mouse zygotic genes does not occur until the 2-cell stage, transcription begins in late 1-cell embryos. This is where α-amanitin-sensitive RNA synthesis is first detected by incorporation of labeled nucleotides (22, 23) or by detection of specific mRNAs and proteins (18, 24). Moreover, transplantation of nuclei from 2-cell stage embryos back into 1-cell embryos reveals that late 1-cell embryos can support transcription once ZGA has been initiated (25). Translation of plasmid-encoded genes can also be detected in late 1-cell embryos (26, 27), although most of it does not occur until the 2-cell stage (40–44 h post-hCG in Fig. 1) (17, 20).[2]

Translation of nascent mRNA appears to be delayed until the 2-cell stage, suggesting that the zygotic clock regulates translation as well as transcription. Expression of one transgene was not detected until 10 h after its mRNA first appeared (24), and expression of luciferase activity from a plasmid injected into S-phase-arrested 1-cell embryos was not detected until ~12 h after the appearance of luciferase mRNA.[2] In contrast, luciferase activity appeared coincident with its mRNA when DNA was injected into arrested 2-cell embryos. Delayed translation may result from failure to export nascent mRNA to the cytoplasm (23) or mRNA instability in 1-cell embryos (28). The net result is that transcription is delayed until ~14 h post-fertilization and translation until ~24 h (Fig. 1).

The zygotic clock is not simply the time required to convert sperm and egg chromatin into a transcribable form but a mechanism that involves trans-acting factors that are either required for transcription or suppress transcription. Since RNA polymerase I-, II-, and III-dependent promoters follow the same time course when injected into S-phase-arrested 1-cell embryos,[2] the zygotic clock may regulate the activity of a general transcription factor such as the TBP that is required by all three polymerases (29). This regulation may occur through post-translational modification of the target protein(s), because inhibitors of translation do not prevent transcription of either zygotic genes (30) or plasmid genes.[2] Protein kinase activity may be involved because ZGA is sensitive to specific inhibitors of protein kinase A (13). In *Xenopus* embryos, the absence of functional TBP delays transcription of some promoters until the "midblastula transition" (31, 32).

One advantage of the zygotic clock is to delay ZGA until chromatin can be remodeled from a condensed meiotic state to one in

* This minireview will be reprinted in the 1995 Minireview Compendium, which will be available in December, 1995.

[1] The abbreviations used are: TBP, TATA box binding protein; ZGA, zygotic gene activation; TAF, TBP-associated factor.

[2] J-Y. Nothias, M. Miranda, and M. L. DePamphilis, manuscript in preparation.

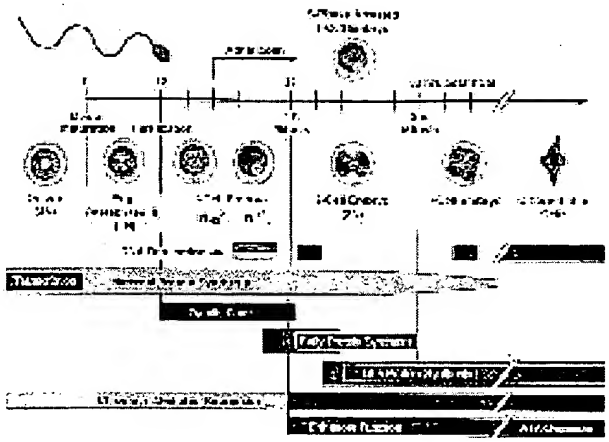

FIG. 1. **Activation of zygotic gene expression.** Events at the beginning of mouse development (13–20) are represented relative to the time after injection of human chorionic gonadotropin (post-hCG), a hormone used to induce ovulation. Except for transcription (*blue bars*), events associated with the paternal pronucleus are indicated in *green*, the maternal pronucleus in *yellow*, and zygotic nuclei in *red*. Addition of aphidicolin to 1-cell embryos prior to the appearance of pronuclei arrests development at the beginning of S-phase but does not prevent the "zygotic clock" from activating "early protein synthesis" or expression of injected plasmid-encoded genes. Chromatin-mediated repression is evident when promoters are injected into the maternal nucleus of oocytes, activated eggs, or fertilized eggs and into the zygotic nuclei of developing 2-cell embryos. The ability to utilize enhancers does not appear until formation of a 2-cell embryo. Stimulation of promoters by an enhancer or transactivator does not require a TATA box until cell differentiation is evident.

which selected genes can be transcribed. Since the paternal genome is completely packaged with protamines that must be replaced with histones, some genes might be prematurely expressed if ZGA were not prevented. Cell-specific transcription requires that newly minted zygotic chromosomes repress most, if not all, promoters until development progresses to a stage where specific promoters can be activated by specific enhancers or transactivators.

Repression at the Beginning of Mammalian Development

The transition from a 1-cell to a 2-cell mouse embryo is marked by the appearance of repression that reduces the activity of any promoter (6, 17, 20, 33–35)[2] or replication origin (36) injected into either embryo from 20- to >500-fold. This repression is produced sometime between S-phase in a 1-cell embryo and formation of a 2-cell embryo and increases as development proceeds to the 4-cell stage (35). Repression is not observed when DNA is injected into the paternal pronucleus in an S-phase-arrested 1-cell embryo; the activities of both promoters and replication origins injected under these conditions are equivalent to their enhancer-stimulated activities in 2-cell embryos. However, repression is observed when DNA is injected into the maternal pronucleus of a 1-cell embryo, parthenogenetically activated egg, or growing oocyte (20, 33). Therefore, the maternal pronucleus appears to inherit its repression activity from the oocyte. The fact that transplantation of an injected paternal pronucleus from a 1-cell to a 2-cell embryo represses the injected gene (35) confirms that repression is absent from the cytoplasm of early 1-cell embryos rather than simply excluded from paternal pronuclei. Repression in 2-cell embryos can act on any nucleus, regardless of its parental origin or ploidy. Two-cell embryos constructed to contain only maternal or paternal nuclei with one or two sets of chromosomes were equivalent to 2-cell embryos with zygotic nuclei in terms of their ability to repress an injected gene (33). Moreover, repression occurs in 2-cell and 4-cell embryos regardless of whether or not these embryos continue development or are arrested in S-phase under the same conditions used to arrest 1-cell embryos. Therefore, the absence of repression in paternal pronuclei in S-phase arrested 1-cell embryos is neither unique to S-phase nor to experimental conditions.

Treatment of mouse embryos with butyrate suggests that repression is mediated through chromatin structure. Butyrate inhibits histone deacetylase, thereby inducing hyperacetylation of core histones, which increases the accessibility of DNA to transcription factors and reduces the ability of nucleosomes to interact with histone H1 (37, 38). Plasmid DNA injected into mouse ova is assembled into chromatin (20, 28). Butyrate relieves repression of this DNA in the maternal nuclei of oocytes, activated eggs, and 1-cell embryos, as well as in 2-cell embryos regardless of nuclear origin or ploidy, but butyrate does not stimulate promoter activity in the paternal pronuclei in 1-cell embryos where repression is not observed (33, 34). Furthermore, butyrate does not change the pattern of endogenous protein synthesis. Thus, butyrate appears to stimulate plasmid gene expression by altering its chromatin structure rather than by increasing synthesis of transcription factors which would activate promoters injected into either pronucleus.

Changes in chromatin structure may result from changes in the levels of histone H1 and the acetylated state of core histones. Incorporation of labeled amino acids reveals that histone H1 synthesis begins in late 1-cell embryos,[3] although histone H1 is not detected by antibodies until the late 4-cell stage (40). Since early histone synthesis is insensitive to α-amanitin and the antibodies were made against somatic histones, these data likely reflect two histone pools, maternal and zygotic. Binding of histone H1 to chromatin leads to chromatin condensation with concomitant repression of transcription (41). In transcriptionally active genes, this repression is countered by acetylation of core histones, because histone H1 binds poorly to hyperacetylated chromatin (37, 38). Fractionation of nascent histone H4 by gel electrophoresis and staining of embryos with antibodies against acetylated H4 reveal that core histones are hyperacetylated in 1-cell embryos and deacetylated as 2-cell embryos proceed to the 4-cell stage.[3] Therefore, the repression that appears concurrently with ZGA could result from the onset of histone H1 synthesis with concomitant core histone deacetylation (Fig. 2). Repression in maternal pronuclei could result from maternally inherited histone H1.

Acquisition of Enhancer Function

Enhancers provide one mechanism that can overcome chromatin-mediated repression. Promoters consist of transcription factor binding sites located upstream and proximal to the transcription start site, while enhancers consist of transcription factor binding sites distal to the start site that are located in either orientation upstream or downstream of the promoter. Enhancers impose tissue specificity on promoter activity. The ability of enhancers to stimulate promoters during mouse development is not observed until formation of a 2-cell embryo; plasmids injected into growing oocytes or S-phase-arrested 1-cell embryos require a promoter to express a gene, but the promoter is not stimulated by enhancers that function efficiently in 2- and 4-cell embryos (5, 17, 20, 33, 34, 42) (Fig. 1). A similar result is observed with the polyoma virus replication origin (36). Arresting 2- or 4-cell embryos at the beginning of their S-phase under the same conditions used to arrest 1-cell embryos does not affect their ability to utilize enhancers.

A survey of polyoma virus mutants that replicate in undifferentiated mouse embryonal carcinoma or embryonic stem cells identified the F101 polyoma virus enhancer as the most effective in stimulating the activity of promoters injected into 2-cell mouse embryos (20, 42). Stimulation ranges from 20- to >300-fold (17, 20, 33, 34, 42). Its activity depends on DNA binding sites for transcription factor TEF1 (42) and on cellular transcription factors that can be depleted in competition experiments (20, 36). TEF1 is a highly conserved transcription factor in mammals and the prototype of the gene family consisting of three or four proteins that share the same TEA DNA binding domain (43, 44).[4] Recent studies using *in situ* hybridization and injection of a TEF1-dependent synthetic promoter suggest that the TEF1 gene family is not expressed until ZGA.[4] Since TEF1 itself is not required for preimplantation development (46), another member of this family may activate enhancers in preimplantation embryos.

The ability to use enhancers is not dependent on formation of a zygotic nucleus, because stimulation by enhancers also occurs in 2-cell embryos constructed with nuclei derived exclusively from either the maternal or paternal pronucleus (33). Moreover, the

[3] M. Wiekowski, M. Miranda, B. M. Turner, and M. DePamphilis, unpublished data.

[4] K. Kaneko, E. Cullinan, M. Miranda, and M. DePamphilis, manuscript in preparation.

F101 enhancer is active if injected into a 1-cell embryo, and the injected pronucleus is then transplanted to a 2-cell embryo (35). Conversely, the F101 enhancer is inactive if injected into a 2-cell embryo, and the injected zygotic nucleus is then transplanted to a 1-cell embryo (35). Therefore, the ability to utilize these enhancers must depend on one or more factors that are not available until formation of a 2-cell embryo.

This hypothesis was tested using plasmids containing a tandem series of yeast GAL4 DNA binding sites located either proximal to the transcription initiation site (GAL4-dependent promoter) or distal to the HSV thymidine kinase promoter (GAL4-dependent enhancer). Each plasmid was co-injected together with an expression vector for GAL4:VP16 protein (34).[5] In the presence of sufficient GAL4:VP16 protein to drive the GAL4-dependent promoter at its maximum rate, the GAL4-dependent enhancer strongly stimulated promoter activity when injected into 2-cell embryos but not when injected into oocytes or into either pronucleus of S-phase-arrested 1-cell embryos. Therefore, enhancer function requires a co-activator that is not available until formation of a 2-cell embryo, presumably because it is expressed during ZGA (Fig. 1). This enhancer-specific co-activator may be a TBP-associated factor (TAF) (48), but it must differ from the TAF that mediates interaction between the basal level transcription complex and GAL4:VP16 bound proximal to the transcription start site. Transcription factors can have multiple activation domains whose function depends on their proximal or distal location to the transcription start site (49). Each domain may interact with a different TAF.

Most, perhaps all, promoters that are stimulated by enhancers contain a TATA box. The TATA box binds the basal level transcription complex through its TBP and determines the direction and start site for transcription (50). There are at least 12 examples of eukaryotic promoters that exhibit TATA-dependent stimulation by enhancers or transactivators, suggesting that a major role of the TATA box is to mediate promoter stimulation by an enhancer (Ref. 7 and references therein). Therefore, it is not surprising that disruption of the HSV thymidine kinase promoter's TATA box element does not affect its efficiency in differentiated mouse cells unless the promoter is stimulated by an enhancer or its natural transactivator, HSV ICP4 (7). Presumably, this stimulation is mediated through TBP. However, it is surprising that this TATA box is not required for promoter activity or stimulation of the promoter by an enhancer or transactivator in cleavage stage mouse embryos and embryonic stem cells (7). Instead, enhancer stimulation of the thymidine kinase promoter in these undifferentiated cells is mediated through transcription factor Sp1. Thus, there appears to be a developmental switch that changes the pathway through which promoters are stimulated by enhancers. This switch could provide a simple mechanism for early embryos to utilize enhancers or transactivators to stimulate the activity of promoters that lack a TATA box but that contain one or more binding sites for Sp1, and then, following cell differentiation, reduce the activity of the same promoter to its basal level. "Housekeeping genes" (genes expressed ubiquitously and at low levels in differentiated cells) frequently are driven by TATA-less promoters containing Sp1 sites and therefore are candidates for this type of developmental control.

The primary role of enhancers is not simply to provide additional transcription factors to facilitate formation of an active initiation complex but to relieve repression of weak promoters from chromatin structure. Enhancers and butyrate appear to overcome the same problem. For example, the capacity of oocytes, S-phase-arrested 1-cell embryos, and 2-cell embryos to utilize a plasmid-encoded promoter is essentially the same in the presence of butyrate (33). In 2-cell embryos, these high levels of activity also can be achieved by linking the promoter to an embryo-responsive enhancer (34). Furthermore, the need for enhancers in 2-cell embryos does not result from functional changes in the promoter elements recognized by the transcription complex, because the thymidine kinase promoter depends on the same transcription factor binding sites in S-phase-arrested 2-cell embryos as in S-phase-arrested 1-cell embryos (34). Moreover, enhancers do not compensate for low concentrations of transcription factors needed to activate promoters, because transcription factor Sp1, which is required for thymidine kinase promoter activity, is 4–6-fold more abundant in S-phase-arrested 2-cell embryos where full activity of this promoter requires an enhancer than in S-phase-arrested 1-cell embryos where it does not (34, 51). In fact, enhancers stimulate promoters in cell-free systems only when the DNA is packaged into chromatin containing histone H1 (41). Thus, the requirement for enhancers in 2-cell embryos may result from changes in chromatin structure that accompany ZGA and produce a general repression of promoter activity.

[5] S. Majumder and M. DePamphilis, unpublished data.

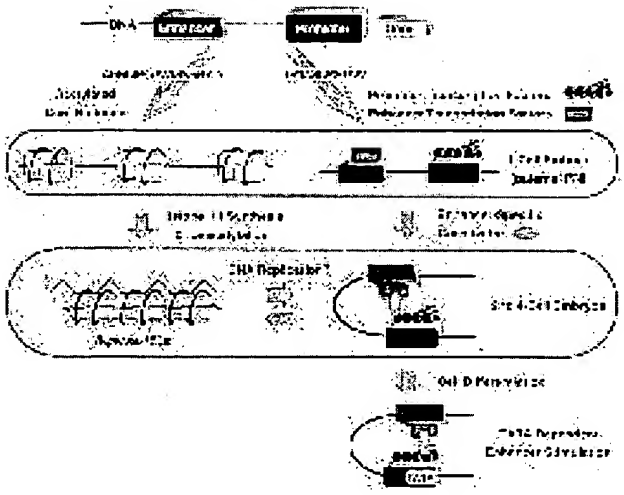

FIG. 2. **Repression *versus* activation.** Genes that are injected into the nuclei of oocytes or cleavage stage embryos are either repressed by chromatin assembly or transcribed by formation of an active transcription complex. A similar choice affects replication origins. We suggest that DNA replication is required to reprogram a DNA molecule that is assembled into either a repressed or activated state.

A Role for DNA Replication in Activation of Zygotic Gene Expression

Enhancers alone cannot always relieve chromatin-mediated repression. Once a repressed state is formed, it may be necessary for DNA to replicate in order to reprogram itself into a transcriptionally active state (Fig. 2). When DNA is injected into either pronucleus of 1-cell embryos and the injected embryo then undergoes mitosis to form a 2-cell embryo, the injected promoter becomes "irreversibly" repressed, in that neither enhancers nor butyrate restores its activity (33, 35). This is not due to loss of plasmid DNA from the injected pronucleus during mitosis, because repression is reversible when the injected pronucleus is transplanted to a 2-cell embryo that then undergoes mitosis (35). Therefore, something happens to DNA between completion of S-phase in a 1-cell embryo and formation of a 2-cell embryo that prevents activation of injected genes, while allowing embryonic genes to undergo ZGA. One explanation is that plasmid DNA does not replicate when injected into mouse embryos unless it contains a viral replication origin (52), whereas the genome of a 1-cell embryo undergoes one round of replication prior to early ZGA and two rounds prior to late ZGA. DNA replication may be required to restore the newly remodeled zygotic genome to a transcriptionally competent state. Chromatin assembly in 1-cell embryos occurs in the absence of at least one factor required for enhancer function that does not appear until the 2-cell stage ("enhancer specific co-activator", Fig. 2). Therefore, if chromatin-mediated repression begins in late 1-cell embryos, before enhancers are functional, DNA replication may be required to disrupt the repressed state so that appropriate transcription factors can bind (53, 54). Conversely, once an enhancer has acted to prevent repression of its adjunct promoter, the resulting transcription complex may remain active until replication again allows reprogramming. Thus, the fraction of genes encoded by plasmid DNA that are "on" or "off" will depend on the relative amounts of repressor *versus* enhancer activation proteins present at the time of injection.

Summary

The maternal to zygotic transition can be viewed as a cascade of events that begins when fertilization triggers the zygotic clock that delays early ZGA until formation of a 2-cell embryo. Early ZGA, in turn, appears to be required for expression of late ZGA, and late ZGA is required to form a 4-cell embryo. ZGA in mammals is a time-dependent mechanism rather than a cell cycle-dependent mechanism that delays both transcription and translation of nascent transcripts. Thus, zygotic gene transcripts appear to be handled differently than maternal mRNA, a phenomenon also observed in *Xenopus* (55). The length of this delay is species-dependent, occurring at the 2-cell stage in mice, the 4–8-cell stage in cows and humans, and the 8–16-cell stage in sheep and rabbits (4). However, concurrent with formation of a 2-cell embryo in the mouse and rabbit (47, 56), perhaps in all mammals, a general chromatin-mediated repression of promoter activity appears.

Repression factors are inherited by the maternal pronucleus from the oocyte but are absent in the paternal pronucleus and not available until sometime during the transition from a late 1-cell to a 2-cell embryo. This means that paternally inherited genes are exposed to a different environment in fertilized eggs than are maternally inherited genes, a situation that could contribute to genomic imprinting. Chromatin-mediated repression of promoter activity prior to ZGA is similar to what is observed during *Xenopus* embryogenesis (31, 32) and ensures that genes are not expressed until the appropriate time in development when positive acting factors, such as enhancers, can relieve this repression. The ability to use enhancers appears to depend on the acquisition of specific co-activators at the 2-cell stage in mice and perhaps later in other mammals (47, 56), concurrent with ZGA. Even then, the mechanism by which enhancers communicate with promoters changes during development (Fig. 2), providing an opportunity for enhancer-mediated stimulation of TATA-less promoters (*e.g.* housekeeping genes) early during development while eliminating this mechanism later during development.

The net result of this sequence of events is to impose a directionality at the very beginning of animal development. This directionality is evident from the inability of fertilized mouse eggs to reprogram gene expression in nuclei taken from cells at developmentally advanced stages. For example, nuclei transplanted from mouse embryos that have progressed beyond ZGA (>late 2-cell stage) into enucleated 1-cell embryos do not recapitulate the normal program of gene expression (45) and therefore do not support successful development (21, 39). At least two factors contribute to this phenomenon: the inability of 1-cell embryos to relieve repression once it has been established and their inability to utilize enhancers. Although S-phase-arrested 1-cell embryos can efficiently utilize promoters encoded in plasmid DNA, they cannot relieve repression of the same promoter if it is first injected into a 2-cell embryo and then the injected nucleus transplanted back into an arrested 1-cell embryo (35). Linking the promoter to the F101 enhancer does not stimulate activity under these conditions, presumably because enhancer-specific coactivator is absent in 1-cell embryos (Fig. 2). Thus, it is not surprising that the maternal pronucleus in 1-cell embryos can exist in a repressed state while the paternal pronucleus does not (33)[2] (Fig. 1).

The results described above have opened the door to understanding how the developmental program in mammals is initiated. It should now be possible to identify the roles of specific transcription factors and chromosomal changes in activating specific genes at the beginning of mammalian development.

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